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NEW STRATEGIES FOR PROTEIN CRYSTAL GROWTH

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■ **Abstract** Protein crystallization is the most difficult and time-consuming step in the determination of a protein's atomic structure. As X-ray diffraction becomes a commonly available tool in structural biology, the necessity for rational methodologies and protocols to produce single, high-quality protein crystals has come to the forefront. The basics of protein crystallization conform to the classical understanding of crystallization of small molecules. Understanding the effect of solution variables such as pH, temperature, pressure, and ionicity on protein solubility allows the proper evaluation of the degree of supersaturation present in protein crystallization experiments. Physicochemical measurements such as laser light scattering, X-ray scattering, X-ray diffraction, and atomic force microscopy provide a clearer picture of protein crystal nucleation and growth. This ever deepening knowledge base is generating rational methods to produce protein crystals as well as means to improve the diffraction quality of such protein crystals. Yet, much remains unclear, and the protein crystallization research community will be quite active for many years to come.

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INTRODUCTION

This review article focuses on the crystallization of one particular class of chemical compounds, namely the proteins. Early studies of protein crystallization were pivotal in establishing that enzymes are proteins (27). Because of their use of protein crystallization in purification and classification of biological chemicals, Sumner, Northrop, and Stanley were awarded the Nobel Prize for Chemistry in 1946. Since those early days, protein crystallization has become a critical scientific tool. Crystallization is a simple yet effective means of purifying one protein from a mixture and has found some industrial applications. In addition, crystallization of proteins within living organisms seems to be linked to a variety of diseases. However, the most prevalent use for protein crystallization today is the production of single, high-quality crystals for use in atomic-structure determination via X-ray diffraction.

The knowledge of the native structure of a given protein is essential both for practical applications such as drug discovery and for fundamental biochemical and molecular biology studies. Structure-based drug design relies on molecular-level structural knowledge of a disease-associated receptor or ligand. Such targets are usually proteins, allowing the living organism to take advantage of the cascade effect inherent in many enzymatic processes. After the protein target is identified and its structure determined, a chemical is specifically designed to bind this target irreversibly and thus negate its activity. Such a structure-based drug design minimizes the extensive screening typically used to identify an appropriate drug. For example, many years of screening and testing have not yielded an effective drug for influenza. The Spanish influenza virus of 1918 killed >20 million people, and

such worldwide influenza epidemics remain a threat today (53). Classical protein chemistry relies on developing drugs that effectively bind conserved regions of the key viral protein, neuraminidase, but this approach did not yield an effective drug for treating influenza. Peter Colman and coworkers determined the atomic structure of neuraminidase in 1983, which quickly revealed the active site of the molecule (21). Drugs that plug this site (so-called plug drugs) were quickly synthesized and tested. Zanamivir (Glaxo Wellcome) and GS 4101 (Gilead and Hoffman-LaRoche) have proven effective in combating a variety of influenza strains and will soon be available as prescription drugs. On a more fundamental level, understanding the relationship between the structure of a protein and its function in vivo leads to a better fundamental understanding of cellular mechanisms.

Two methods are now commonly used to determine the atomic structural models for proteins, nuclear magnetic resonance and X-ray crystallography. Although nuclear magnetic resonance allows the direct, noninvasive determination of protein structure, it is hampered by low sensitivity and is limited to proteins less than ~30,000 molecular weight (7). X-ray crystallography is not limited in these respects but does require the formation of a single, high-order, three-dimensional crystal. The crystal used for X-ray diffraction experiments has a significant influence on the utility of the method. The generation of a crystal of sufficient size (typically 0.1 mm in all dimensions) is usually a time-consuming task that relies heavily on empirical screening studies. In addition, the crystals must possess high internal order. Measurement of order in protein crystals is not trivial, but the most important measure is the resolution limit of the collected data. High-resolution data (i.e. diffracting to Bragg spacings of 2.0 Å or less) are required to distinguish individual atoms within the large protein structure. Although large, high-quality crystals are essential for structural determination, there are no hard and fast rules for success of their growth. In fact, protein crystal growth is an area of quite active research and will likely see major advances in the near future.

Protein crystallization has traditionally been treated as more difficult than and certainly different from crystallization of small inorganic or organic molecules. This viewpoint was promoted by the biochemists who have focused on the differences, rather than striving to identify the commonalities. Protein crystallization is now seen as very similar to most other chemical systems (75). The only notable differences for protein crystallization systems are the requirement of very high supersaturation to induce nucleation and subsequent growth (relative supersaturations of 100–500% are not uncommon) and the high solvent content of the crystals, which renders them fragile objects. This review focuses on aspects of protein crystallization that are relevant to X-ray crystallography. Thus, the desire for producing single, large, high-quality crystals is a recurring theme in this article. A review of the basic steps required by an X-ray crystallographer to obtain a structure set the stage for subsequent presentation of the variables affecting solubility, nucleation, and growth mechanisms; typical physicochemical measurements; and typical means of screening crystallization conditions. The article concludes with methodologies for evaluating and improving crystal quality.

BRIEF OVERVIEW OF PROTEIN STRUCTURE DETERMINATION VIA X-RAY DIFFRACTION

It is beyond the scope of this short review article to fully describe all of the steps required for determining a protein structure to atomic resolution. There are many excellent texts and monographs that attempt to provide such a comprehensive overview (4, 15, 28, 38, 39, 67, 69, 97). However, the major steps towards structure determination must be described to the reader, if a true appreciation of the importance of growing protein crystals is to emerge.

Step One: Grow a Single, Large Crystal of the Candidate Protein

The protein crystallographer is primarily concerned with identifying one magical solution condition or "gizmo" that will give a relatively large ($\geq$0.1 mm in all dimensions) crystal that diffracts to high resolution. Rarely, if ever, are fundamental studies of solubility, nucleation, and growth kinetics conducted to aid in the process of generating high-quality protein crystals. This lack of a fundamental approach to crystallization is driven by the lack of material (usually only a few milligrams of protein are available) as well as tremendous pressure to get a crystal, get an X-ray data set, get a structure, and get a publication.

For these reasons, protein crystallization will continue to rely on screening techniques that use micromethods. Typical methods allow one to screen >100 solution conditions with just a few milligrams of protein. The choice of which solution conditions to screen has been greatly simplified through the years. Gilliland & coworkers (37) publish a database of crystallization conditions which is extremely helpful. This Biological Macromolecular Crystallization Database is available at no cost via the Internet (http://wwwbmcd.nist.gov:8080/bmcd/bmcd.html). Jancarik & Kim (46) proposed that most protein crystals are produced by a relatively limited number of precipitants, and they developed a sparse sampling matrix that efficiently sampled a variety of precipitants and pH. Screening kits based on this sparse matrix approach, as well as a variety of other useful solution conditions, can be purchased in ready-to-use form (for example, see http://www.hamptonresearch.com)

If crystals are produced during the screening trials, further optimization is conducted, and many crystals are produced at the optimized condition. If crystals are not produced, the screens are usually moved to a cold room and allowed to re-equilibrate. Ultimately, if crystals are still not evident, the system is deemed uncrystallizable, and the scientist moves on to other more promising candidate proteins. Unfortunately, the failures are never reported in the literature, and it is likely that other scientists are wasting time reproducing these same failures. Without crystals, the X-ray diffraction experiment cannot proceed, and the structure will not be obtained.

Step Two: Mount the Crystal

Typically, one-third to one-half of a protein crystal's weight is from water present within the crystal itself. The molecular contacts within the crystal are fairly weak, consisting of hydrogen bonds and weak ionic interactions. Thus, a sharply faceted protein crystal that looks very similar to a diamond (see Figure 1, typical lysozyme crystals) is, in fact, a very fragile object. Moving the crystal from the growth solution to a suitable mounting apparatus is an important step in obtaining the X-ray diffraction data. Fracturing the fragile crystal is a primary concern, but providing a means of hydrating the crystal is equally important.

In 1934, Bernal & Crowfoot (3) conducted an X-ray diffraction experiment on a pepsin crystal suspended in the mother liquor and were the first group to observe a sharp diffraction pattern for a protein with lattice spacings that correspond to atomic distances. Thus, the presence of water is an important component of the crystal, and it must be maintained within the crystal.

Until very recently, protein crystals were typically mounted within thin-walled glass capillaries. This mount requires carefully aspirating a single crystal into a capillary, floating it to a suitable location, and wicking away excess mother liquor. Typically, a small pool of the mother liquor is left in the capillary, which maintains the proper humidity in the sealed capillary. Such capillary mounts can maintain the crystal in a stable state for several weeks without noticeable degradation in the physical or visual properties of the crystal.

Any crystal will deteriorate in the X-ray beam owing to the generation of free radicals by the intense X-ray source and heating by the beam. The high-intensity X-rays are absolutely necessary to measure the intrinsically weak diffracting high-

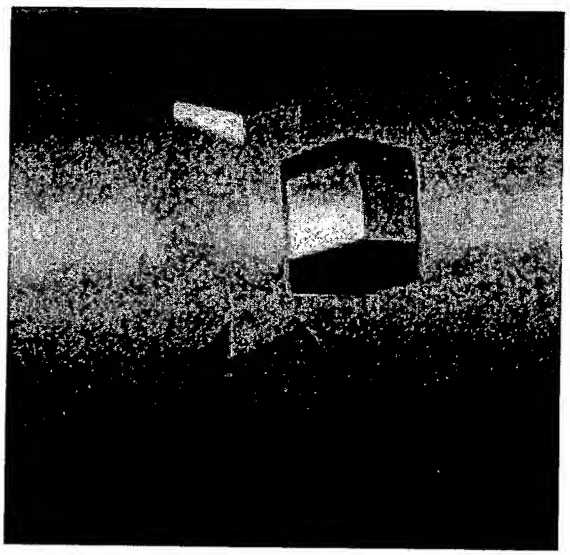

FIGURE 1 Some typical protein crystals of lysozyme. Note the optical clarity and sharply faceted surfaces of the crystal, preliminary indications of high quality.

resolution peaks needed to map the atomic coordinates of large molecules such as proteins. Traditionally, the diffracting crystal is occasionally replaced with a new crystal once the radiation damage is significant. This approach is problematic for two reasons. First, 10–50 crystals are required to collect a full, high-resolution data set. This large number of valuable crystals may not be available. The second problem is the merging of data sets obtained from different crystals. Such data merging mandates that some reflections be collected multiple times (several times per crystal) to allow scaling of the multiple data sets. This reduces the collection efficiency of the X-ray diffraction experiments and introduces additional experimental error into the final structural model of the protein owing to the slight variations from crystal to crystal. The preferred option is to collect all of the needed data with one crystal. It has been known for many years that cooling the crystal to liquid nitrogen temperatures should greatly reduce the formation and subsequent movement of free radicals in the crystal. However, protein crystals typically contain 50% water by mass. Initial attempts at cooling (56) resulted in ice formation and increased peak widths (called mosaic spread).

Within the past 7 years, protein crystallographers have developed techniques of quickly cooling crystals to liquid nitrogen temperatures, which allows the collection of complete data sets on one crystal (35, 45, 96). It is now possible in many cases to quickly cool the crystal and produce an amorphous or vitrified form of water within the protein crystal. This method of crystal mounting uses fine loops to pluck the crystal out of the mother liquor, which is then quickly cooled in a gaseous nitrogen stream, typically at a temperature of 100 K. The cold temperature not only stabilizes the water in the crystal, it also minimizes the radiation damage typically seen with room temperature crystals. This vitrified water shows no strong diffraction rings or spots in the collected data sets in many cases. Data collection commences and is usually completed on the same crystal. With the advent of cryocrystallography, most crystallographers are now interested in using extremely high-intensity X-ray sources, which are available at a variety of synchrotron facilities throughout the world.

Step Three: Collect and Evaluate Preliminary Diffraction Data

This step is typically the moment of truth for the candidate crystal. It is not uncommon to find an optically clear, highly faceted crystal that will not diffract X-rays. This indicates a lack of long-range order within the crystal, and a new crystallization condition must be identified (back to step one). There are a variety of diffraction instruments that can be used to collect sufficient data to judge crystal quality and provide estimates of the space group. Most modern laboratories use a collimated X-ray beam from a rotating anode detector, a single phi-axis goniostat, and an area detector for measuring the location and intensity of diffracted X-rays (called reflections). The data are recorded as simple digital images in a desktop computer.

Owing to the fixed geometry of the crystal and detector, the images contain sufficient information to allow the determination of the molecular packing within the crystal or its space group. This process is referred to as indexing, and most commercial vendors of detectors supply such software to aid in this process. Beyond indexing, this preliminary data can be evaluated for the limits of diffraction. Visual inspection of the diffraction images will indicate the limits of diffraction. Reflections near the edge of the detector are high-resolution spots and are necessary for atomic-level resolution in the protein structure. Figure 2*a* shows a high-resolution data set of lysozyme with a resolution overlay. This crystal has reflections well beyond the 2.0-Å Bragg spacing, whereas the crystal shown in Figure 2*b* diffracts only to low resolution (~4.5 Å).

The space group and the limit of diffraction are critical pieces of information to develop a data collection strategy. To accurately calculate an atomic structure, essentially all predicted reflection intensities must be recorded. Using Bragg's law, Laue relations, and the Ewald sphere construction, one can calculate all possible reflection locations for a given space group and limit of diffraction. In addition, space groups have various degrees of symmetry. A highly symmetric lattice such as a cubic lattice will produce a highly symmetric diffraction pattern. Thus, a limited portion of the Ewald sphere of reflections will contain all of the information needed because all other reflections can be obtained by applying symmetry relationships. Thus, a data collection strategy can be developed that

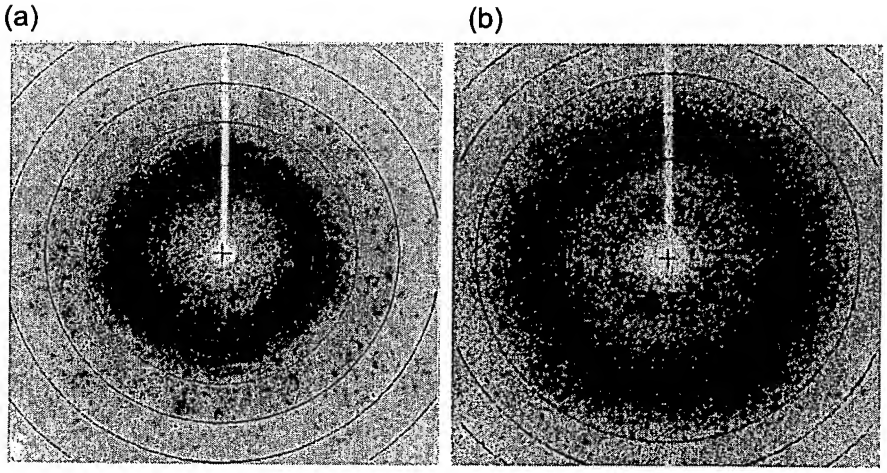

FIGURE 2 Oscillation images of diffraction reflections for (*a*) lysozyme and (*b*) hemoglobin. Note that the spots (properly called reflections) extend further away from the direct beam (denoted with crosshairs) for lysozyme versus hemoglobin. Circular arcs indicate the resolution of the reflection. The resolutions of each arc from the innermost to the outermost arc are 4.3 Å, 3.0 Å, 2.5 Å, 2.1 Å, and 1.9 Å. The lysozyme will yield a high-resolution data set (~2.0 Å) versus the low resolution observed in the hemoglobin crystal (~4.5 Å).

will yield the maximum amount of nonredundant data. At this point, the crystallographer is ready to collect a complete data set on the crystal.

Step Four: Complete Data Collection and Estimate Phases

In 1934, after Bernal & Crowfoot (3) successfully collected a sharp diffraction pattern from a pepsin crystal, the determination of the atomic structure was still beyond the capabilities of crystallographers, with the phase problem looming as the major stumbling block. The X-ray diffraction experiment yields the electron density of the molecule from which the atom coordinates are derived. The electron density of the molecule is calculated from its Fourier series representation:

$$\rho(x,y,z) = \frac{1}{V}\sum_h\sum_k\sum_l \mathbf{F}_{hkl}e^{-2\pi(hx+ky+lz)}, \quad (1)$$

where x, y, and z are the atomic coordinates; ρ is the electron density; V is the volume of the unit cell; h, k, and l are the Miller indices of the summation; and $\mathbf{F}_{hkl}$ (a vector quantity) is the structure factor for reflection hkl. The amplitude of the structure factor is equal to the square root of the measured intensity of reflection hkl. In addition to the amplitude, the structure factor includes information about the relative phase of the reflection, which cannot be measured in a direct fashion. This missing information is critical to determining the three-dimensional electron density and, thus, the protein's atomic structure. Perutz et al, after 16 years of working on the phase problem, postulated the now commonly used multiple isomorphous replacement technique in 1954 (41). The first atomic structures of myoglobin (48) and hemoglobin (71) soon followed.

Phases can also be estimated by using a model protein structure (e.g. the wild-type protein in mutation studies) as a starting point. In this case, the problem can be reversed, and structure factors calculated for the proposed structure, including phases. The calculated amplitude of the structure factor can be compared with the measured reflection intensity to yield a goodness-of-fit statistic. The model protein structure is then translated and rotated until the goodness-of-fit statistic is optimized.

With the availability of high-energy synchrotron X-ray radiation, some rather subtle differences in reflection intensities owing to absorbance of X-rays by some of the protein's constituent atoms can be used to estimate phases without the need to have heavy-atom derivatives or a model structure. Referred to as anomalous dispersion or anomalous scattering, the technique was not exploited until recently because most laboratory X-ray sources do not allow a tuning of the X-ray wavelength. At synchrotron sources, the X-ray wavelength can be tuned over a wide range of values, and the relative reflection intensities can be recorded at multiple wavelengths. The best strategies to date use proteins expressed with selenium in place of sulfur. The selenium has ideal X-ray absorbance spectra to yield measurable changes in reflection intensities as a function of the X-ray wavelength.

The technique is currently very popular for phase estimation and is commonly referred to as multiple wavelength anomalous dispersion.

For small molecules, phases are estimated from very high-resolution data (beyond 1.0 Å) and relationships imposed by the requirements that all electron density must be positive. Such techniques have recently been applied to proteins (28) but will probably not gain popularity in view of the requirement for data of high resolution—typically beyond that achieved for most proteins.

Having a native data set of intensities and estimates for phases, the crystallographer can now leave his laboratory and focus on computations and graphics. Of course, as the calculations and graphical construction of the structure proceed, it may become evident that some critical part of the structure is missing, and another trip to the laboratory to grow more crystals may be in order.

Step Five: Trace and Refine Chains

The estimates for structure factor amplitudes and phases are substituted into Equation (1), and the electron density map is calculated in three dimensions. At this point, a graphics program such as O (47) or Xtalview (69) is used to visualize the skeletonized representation of the electron density (i.e. a three-dimensional surface of constant electron density). The challenge is to find one end of this extended tube and to start placing the appropriate amino acids into the electron density map.

After the initial graphical tracing of the protein backbone and side chains, the estimated coordinates of the protein molecule are passed on to a refinement program. TNT (91) and CNS (10) are two commonly used refinement software packages. These packages invoke least-squares or Monte Carlo techniques that adjust atom positions to optimize known stereochemical constraints of adjacent amino acids in the protein (40). Each round of refinement is followed by structure visualization and graphical adjustment of the structure as deemed necessary. The means of assessing the quality of the structure is the R factor. The R factor is basically the normalized sum of square errors between the measured structure factor amplitudes and the calculated structure factor amplitudes. Kleywegt & Brunger (49) outline a procedure to calculate such R factors with data not used during refinement (called R free), which is not biased by overfitting the data.

CURRENT STATE OF THE ART IN STRUCTURE DETERMINATION

Over the past century, the obstacles to protein structure determination have gradually been surmounted. The first problem of obtaining diffraction was addressed by Bernal & Crowfoot (3) by properly mounting the protein crystals to maintain their water content. The second major stumbling block was the phase problem, which was elegantly addressed by Perutz (41) with multiple isomorphous replace-

ment techniques. Relatively small proteins such as myoglobin could be solved to low resolution. The next major obstacle was computing and visualization power. The computer revolution and high-end graphics workstations have set this obstacle aside. The main obstacle that remains is the production of single, large, diffraction-quality protein crystals. Once the crystal is in hand, the probability of obtaining a structure, although not 100%, is very high. Although protein crystallographers and biochemists have traditionally viewed protein crystallization as simply a small hurdle in the process of protein structure determination, it is now evident that it is the main hurdle. For this reason, protein crystallization has become a research subject in and of itself, not simply an extension of the protein crystallographer's laboratory.

VARIABLES AFFECTING PROTEIN SOLUBILITY

As in any crystallization, the production of protein crystals requires bringing the protein into a supersaturated liquid state. Of course, the degree of supersaturation determines the rate of nucleation as well as the crystal growth rate. Each of these phenomena is an important player in the crystal's diffraction quality and morphology. The degree of supersaturation is defined by the equilibrium solubility of the protein. Whatever variable affects the solubility of the protein can also be used to control supersaturation in the system and, thus, indirectly, the rates of nucleation and growth. The thermodynamic variables of temperature, pressure, and composition are typically used to alter the solubility of proteins.

Effect of pH on Protein Solubility

Protein solubility will change dramatically as pH is altered by ~0.5 pH units; however, some systems are sensitive to pH changes as small as 0.1 pH units (98). The protein of interest will often dictate acceptable pH ranges for crystallization. Only pH values that maintain the folded structure of the protein are acceptable conditions for protein crystal growth.

Usually the protein solubility is minimal at the protein's isoelectric point (pI) (74), where its net charge is zero. Many proteins are not stable near their pI and require crystallization at pH values far from the pI, and, thus, pH may not be a significant screening variable in these cases. The useful range of pH for initial screening studies is usually defined by the extreme of pH at which the protein is still stable and the pI of the protein.

Effect of Electrolyte on Protein Solubility

Protein purification has long relied on precipitation via "salting out." The basic idea is that "salting" the solution (adding electrolyte) causes the protein to come "out" as a separate solid phase. The effect of the electrolyte on protein solubility is usually described as salting out or salting in. Salting in implies an increase in

the protein solubility as the electrolyte is added to the solution. Depending on the type of electrolyte used, either or both of these phenomena are observed. Salting out is not well understood. One popular explanation for this effect relies on relative hydration of the protein versus bulk electrolyte. In this model, the electrolyte is assumed to bind bulk water as water of hydration near the ion's surface. Likewise, the protein needs to be hydrated with water. As the electrolyte and the protein compete for bulk water to hydrate their respective surfaces, the protein becomes partially dehydrated and prefers to fill such an exposed surface (dehydrated surface) with other protein molecules, thus facilitating crystal contacts. Therefore, the solubility of the protein is reduced as the electrolyte is added to the protein solution. The effectiveness of ions (cations and anions) to cause phase separation (or lower solubility) has been documented by many investigators and is named the Hofmeister (or lytropic) series (13, 43, 52, 54, 57, 92).

Results indicate a clear trend in the effect of electrolyte type on protein solubility, depending on the pI of the protein. Acidic proteins follow the Hofmeister series for anions, whereas basic proteins follow the reverse of the Hofmeister series for anions. These studies also consistently find that the anion dominates the effect of the electrolyte on the protein solubility. Collins (20) predicts that anions should have a much larger effect based on their ability to more significantly alter the structure of water than cations. In addition, anions are more strongly hydrated than cations for a given charge density.

Effect of Antisolvents on Protein Solubility

Other common chemical additives to reduce protein solubility are small, polar organic molecules such as methanol, ethanol, and acetone. Such water-miscible solvents are often called antisolvents. The most widely known separation using such solvents is the method of blood fractionation from Cohn et al, which uses ethanol for the recovery of a variety of proteins from blood (19). Such separations are usually carried out near the pI to bring the protein to a point of minimal solubility and minimize the amount of antisolvent required to achieve the precipitation or crystallization. Antisolvent addition has multiple effects on the protein. First, these water-miscible solvents tend to act in a manner similar to electrolytes, competing with the protein for water of hydration and "solventing-out" the protein. Second, the solvent tends to lower the dielectric constant of the water, thereby enhancing electrostatic interactions between protein molecules and reducing their solubility. The lower dielectric constant also tends to solubilize the hydrophobic core of the protein molecule, which can lead to protein unfolding or denaturation. Thus, antisolvents are generally added while the protein is kept cold, typically at or below the freezing point of water.

Effect of Soluble Synthetic Polymers on Protein Solubility

PEG is the water-soluble polymer of choice for protein crystallization; however, there is no reason to suspect that other water-soluble polymers would be any less effective. PEG, like small water-miscible organic solvents, will hydrate as well

as alter the dielectric constant of the solvent. However, the volume exclusion effect must account for its effectiveness, especially for the high-molecular-weight PEGs. PEGs of molecular weight 4000 or 6000 seem to be very effective for most protein systems. In general, the higher the molecular weight, the more effective the PEG is at reducing the protein solubility.

Effect of Pressure on Protein Solubility

In 1990, Visuri & coworkers (94) reported that glucose isomerase crystallized rather rapidly at elevated pressures (100 MPa or higher). This initial exciting result motivated a body of work on the effect of pressure on lysozyme solubility and growth kinetics (42, 55, 77, 81, 85, 86). Although this field continues to advance at a rapid rate, it has come to some definite conclusions for lysozyme. Schall et al (81) clearly demonstrated that the rates of growth are dramatically depressed for lysozyme at high pressures, which is in sharp contrast to the results of Visuri et al (94) on glucose isomerase. Subsequent studies (77, 86) provided further quantitative support of this conclusion. The reduced growth is primarily attributed to the increased solubility of lysozyme under high pressures, as shown by Takano et al (86). The discrepancy between the results for lysozyme and glucose isomerase suggests that pressure can have a variable effect on protein solubility and growth. Nevertheless, the effect of pressure is evident only at extremes of pressure, and, for most practical applications, the effect is negligible.

Effect of Temperature on Protein Solubility

Proteins have been documented to crystallize at a wide variety of temperatures, yet most proteins are crystallized at room temperature (25°C) or in a refrigerator (4°C). Most proteins will denature at temperatures above 40°C, so temperature can be varied over only a limited region (i.e. 0–40°C). Protein solubility can increase, decrease, or remain constant as the temperature of the system increases. In addition, the protein can undergo morphological phase transitions as temperature changes (73).

Classical thermodynamics describes the effect of temperature on the solubility of a molecule in solution. If the heat of crystallization, ΔH_{cry}, and solubility, $C_{sat,1}$, at one temperature (T_1) are known, the solubility as a function of temperature can be determined via the van't Hoff equation:

$$\ln\left(\frac{C^*}{C_1^*}\right) = \left(\frac{\Delta H_{cry}}{R}\right)\left(\frac{1}{T} - \frac{1}{T_1}\right), \qquad (2)$$

where C^* is the solubility of the protein in the liquid phase at temperature T and R is the gas constant. Calorimetric measurement of the heat signal on crystallization under isothermal conditions (22, 79) shows excellent agreement between the experimentally determined solubility and the solubility predicted by Equation (2), using the direct measurement of ΔH_{cry}.

NUCLEATION AND GROWTH MECHANISMS

The fundamentals of nucleation and growth of protein crystals have not been of interest to the general protein crystallography community until the last decade or so. Protein crystallographers, in general, have been interested only in obtaining one good crystal of their particular favorite protein. In fact, the methods typically used to grow a few crystals of X-ray diffraction quality are not suited for fundamental investigations of protein crystal nucleation and growth. These methods, which are described in more detail below, are ideal for screening experiments because a wide variety of precipitants are used in a manner such that supersaturation is increased during the course of the experiment to induce nucleation. Unfortunately, once the experiments start, there is no simple way to track the relative protein and precipitant concentrations, let alone measure the supersaturation of the system. Thus, fundamental information on the nucleation rate versus the growth rate of protein crystals is impossible to extract from such screening experiments. Within the last decade, there has been a rapid increase in the number of investigations focused on the fundamentals of protein crystallization. Classical studies of crystal growth (experimental methods described below) have shown that many of the well-developed models for small molecule crystal growth also apply to proteins. More recent studies of protein crystal growth have allowed for the direct visualization, at the atomic level, of the growing crystal face (64–66), using atomic force microscopy (AFM). This exciting development will be discussed in more detail below.

Nucleation of protein crystals typically requires extremely high supersaturation levels. Studies of protein nucleation are limited, with most efforts focused on light scattering as a tool to detect nucleation. Feher & Kam's (31) work set the tone for much of the work that followed. They modeled nucleation in a classical fashion, as a cooperative step-by-step addition of monomers to a cluster. Light scattering was used to monitor the cluster size distribution as a function of time and solution variables. Certainly, the fundamentals of protein crystal nucleation are an area that deserves additional study.

PHYSICOCHEMICAL MEASUREMENTS

Solubility Determination

Solubility curves have traditionally been determined by either crystallization of a supersaturated solution or dissolution of crystals in an undersaturated solution. Suitable solution conditions that produce crystals must be known in advance for both methods. Protein solubility can be determined as a function of many parameters including temperature, salt concentration, salt type, buffer, and pH. Aliquots are removed periodically for concentration determination for up to 6 to 12 weeks after crystals appear. For dissolution experiments, a batch of crystals previously

grown in the appropriate buffer is needed. Crystals are placed in undersaturated solutions and allowed to dissolve. Again, for 6 to 12 weeks, aliquots are periodically removed for concentration determination.

Both methods should converge to the same solubility for each value of the parameter being measured (74). The main disadvantages of these methods for solubility determination are the amounts of protein (typically gram quantities) and time (6–12 weeks) required. The protein may be recovered at the end of the experiment if contamination has not occurred. Cacioppo et al (12) developed a micromethod for protein solubility determinations. This method requires less protein, ~200 mg of protein or less, to determine key regions of a protein's solubility diagram. Berland et al (2) and Broide & coworkers (9) measure solid-liquid phase boundaries for protein solutions by alternate heating and cooling of a crystal. In a similar fashion, AFM has been used to detect the cessation of step formation and the solid-liquid phase boundary (64). As mentioned earlier, the effect of temperature on solubility can be detected by directly measuring the enthalpy of crystallization (22).

Growth Rate Determination

Crystal growth rates are typically measured via video microscopy (30). Pusey has developed a computer-controlled apparatus able to simultaneously monitor the growth rate of up to 40 crystals (72). Typical experiments may require a week or more for growth rate analysis at a fixed solution condition and temperature.

AFM is now used to relate microscopic measurement of step velocity to macroscopic face growth rates (64). Such data can be collected at a very rapid rate but the technique does require some skill as well as access to a research caliber AFM. Likewise, microcalorimetry may be used to extract crystal growth rates very rapidly, provided that the protein's heat of crystallization is sufficient to yield a measurable signal (22). Both of these techniques can provide growth rates over a wide range of conditions within a few days, as opposed to months by more traditional video microscopy techniques.

Measurement of Aggregation and Interaction Potentials Via Scattering Techniques

Scattering techniques, especially laser light scattering, are finding increased use among protein crystal growers. The protein can exist as a fairly uniform and chemically homogeneous solution when assayed by the electrophoretic and chromatographic techniques. However, the screening conditions used to induce crystallization can dramatically alter the physical state of the protein, leading to aggregates of protein that are very polydispersed. There is now a consensus among protein crystal growers that proteins that exist as monodisperse solutions in a single aggregation state are likely to crystallize (33). Conversely, polydispersed aggregation states usually fail to yield useful crystals. The use of dynamic

light scattering for quickly assaying protein crystallization solution conditions has gained favor in recent years. Using very small volumes (~10 μl), the protein crystal grower can mix the protein in a potential growth solution at a very low concentration and then assay for monodispersity. Only those solutions yielding monodisperse aggregates should be further optimized.

Static light scattering has also been used to a great extent to characterize solution conditions suitable for growth of protein crystals. George & Wilson (36) measured the osmotic second virial coefficient (B_{22}) for a wide variety of soluble proteins under conditions that have successfully yielded crystals. They found that the values of B_{22} lie within a fairly narrow window of -0.8×10^{-4} to -9×10^{-4} mol·ml·g^{-2}. Further, they found that conditions that tended to stabilize the protein and prevent it from crystallizing had large positive values for B_{22}, and conditions that resulted in amphorous precipitate had large negative values. The second osmotic virial coefficient is an integral average of the protein-protein interactions (V), as defined by McQuarrie (68):

$$B_{22} = -2\pi \int_0^\infty x^2 (e^{-V(x)/k_b T} - 1)\, dx, \qquad (3)$$

where x is the distance between the two protein molecule centers, k_b is Boltzmann's constant, and T is the absolute temperature. The solution conditions that promote a slight attraction between the two proteins are required to produce crystals. These conditions would produce a slightly negative value of B_{22} by the above definition. Amorphous precipitate results from highly attractive interactions, which corresponds to large, negative B_{22}. Finally, strictly repulsive interactions maintain the protein in a soluble state and correspond to a positive value for B_{22}. Tardieu and coworkers [see Bonnete et al (5), Budayova et al (11), Finet et al (34), and Tardieu et al (87)] have shown that similar measurements of B_{22}, as well as the protein's aggregation state, can be performed via small-angle X-ray scattering.

Neutron scattering is an especially powerful scattering methodology for studying the solution behavior of proteins. Tiede & Thiyagarajan (88, 90) have used the technique to characterize the complex mixture of micelles and micelle-protein complexes in membrane protein crystallization systems. Small-angle neutron scattering is a widely applied scattering technique, and several excellent reviews cover relevant applications to biological systems (18, 32). The particular advantage of small-angle neutron scattering is the ability to adjust the relative contrast of the solvent and the scattering objects so that one can "tune in" on just one scattering object in a complex mixture. For example, with membrane protein systems it is not uncommon to have detergent micelles, PEG polymer, and a protein-detergent complex all simultaneously present in a solution. Proper solvent contrasting can allow one to eliminate the scattering of one or more of these relatively large objects and simplify the analysis of the resulting data.

Atomic Force Microscopy

AFM is a valuable tool for in situ observation of protein crystal growth at the macromolecular length scale. The device uses a sharp tip on a cantilever arm that is scanned over the specimen's surface. The deflection of the cantilever during this scanning is a measure of the surface topology. Durbin & Carlson (29) were able to devise an appropriate chamber for imaging growing lysozyme crystals with an AFM. This work was the first to actually image surface nucleation, spreading, and merging of two-dimensional islands to form successive growth layers on a lysozyme crystal's surface. Growth steps are clearly seen in such images, and the dynamics of the growth steps are easily observed. The presence of two-dimensional nucleation, as well as screw dislocations, is directly visualized and validates the mechanisms postulated based on macroscopic measurement of crystal face growth rates (66). Some example images are shown in Figure 3 (see color figure). This figure shows the surface of a growing crystal of canavalin. Figure 3*a* shows a right- and left-handed screw next to each other near the center of the image, as well as a double screw dislocation in the upper left portion of the image (magnified in Figure 3*b*). On a larger scale, these screw dislocations form spiral growth steps, as shown in Figure 3*c*.

The use of AFM has increased rapidly since 1992. Konnert et al (50) have focused on verifying the crystalline periodicity in the scanned structure and quantifying the effect of the tip on the observable quantities. In general, they conclude that the tip perturbs the surface to a very small extent, thus validating the reported results in the literature. Malkin et al (64) have used the AFM technique to provide a quantitative measurement of microscopic tangential step velocities, which can then be used to calculate macroscopic face growth rates as traditionally measured with an optical microscope. The advantage of such molecular-scale measurements is the ease in quantifying the effect of impurities and environmental parameters such as temperature and solution pH.

SCREENING EXPERIMENTS

For each experiment described below, there is an ideal trajectory that the experiment needs to traverse in the phase diagram of the protein. Typically, the protein concentration and the precipitant concentration are varied simultaneously to move around the phase diagram. Unfortunately, screening experiments typically sample just one or a few starting conditions for a given precipitant and may yield poor or no crystals even though the precipitant may, indeed, be ideal for the protein at hand. Thus, screening is usually far from the optimal solution conditions. Once screening is completed, further optimization is required, and such optimization is amenable to tried-and-true statistical treatments (14).

Vapor Diffusion Experiments

Vapor diffusion experiments rely on water (and other volatile species) evaporation from a small droplet containing the protein and precipitant to slowly increase both the protein concentration and precipitant concentration within the droplet. Although the least controlled method that one can use, vapor diffusion has proven to be the most popular technique for growing protein crystals. This method allows one to simultaneously increase the protein concentration and decrease the protein solubility owing to the increased precipitant concentration. In vapor diffusion, the initial conditions are usually undersaturated. The most commonly used technique is the hanging-drop method, although the same effect is achieved with sitting drops. A small drop (1–10 μl) of a solution containing the protein is typically mixed with an equal volume of the precipitant solution and placed on a microscope cover slip. The cover slip is inverted over a reservoir of precipitant solution. The reservoir solution is protein free and typically at twice the precipitant concentration of the drop. The supersaturation of the protein solution changes over time as the water in the hanging drop equilibrates with the water in the reservoir solution. Thus, the protein concentration and the precipitant concentration in the drop increase, causing nucleation and crystallization.

Free Interface Diffusion

Free interface diffusion is a technique that has a strong analogy to the vapor diffusion technique. First used by Salemne (78), the technique relies on carefully layering the precipitant solution on top of the concentrated protein solution in a capillary. The narrow diameter of the capillary minimizes mixing from natural convection in the system. Thus, the precipitant and protein slowly interdiffuse. As time proceeds, there is a sampling of solution conditions along the axis of the capillary, ranging from very low precipitant and high protein concentrations to the converse. In essence, many combinations of protein and precipitant concentration pairs are sampled locally within one capillary. Thus, many trajectories are sampled simultaneously on the phase diagram. In the ideal situation, the protein is initially at a high concentration and is slowly diluted by the faster moving (small-molecule) precipitant. The precipitant concentration increases to the point that nucleation occurs and the crystals start growing and consuming local supplies of protein.

Dialysis

Dialysis and batch crystallization methods are the most controlled experiments but also the least commonly used techniques for growing protein crystals. Microdialysis chambers are manufactured from plastic cores to accommodate volumes ranging from 10 to 100 μl. Provided that the precipitant is a small molecule like a salt or alcohol, it can easily penetrate the dialysis tubing, and the protein is slowly brought into equilibrium with the precipitant solution. If the precipitant

has a large molecular weight, like PEG 6000, then the precipitant must be placed in the button along with the protein. Water will transfer through the dialysis membrane until the PEG activity is equalized on both sides of the membrane. Dialysis is the best method for screening a variety of solution conditions at a fixed protein concentration.

Batch Growth

Batch techniques require mixing the precipitant and the protein at the onset of the experiment. To minimize evaporation of the small droplets, the mixture is typically overlaid with a water-immiscible oil such as mineral oil (17). For protein crystal growth by batch techniques, the initial solution must be in the labile zone at a given temperature for nucleation to occur. The batch method has become a popular technique for implementing temperature-controlled protein crystallization (80). Batch techniques are more suitable for optimizing protein crystal growth than vapor diffusion because the system variables are more easily controlled. However, converting vapor diffusion experimental conditions to appropriate batch conditions (or vice versa) requires some care. Chayen (16) presents an excellent review of the advantages of each technique, as well as some guidelines for interconversion of the experimental setups.

Seeding Techniques

When protein microcrystals are available, seeding techniques can be used to increase the size of the crystal to a range acceptable for X-ray diffraction studies. An excellent review of seeding techniques typically used for protein crystals is presented by Stura & Wilson (84).

PROTEIN CRYSTAL QUALITY MEASUREMENT

Because much of the significance of protein and other macromolecular crystals is owing to the ability of X-ray crystallography to provide three-dimensional structure in atomic detail, the evaluation of crystal diffraction quality is an essential adjunct to systematic studies of protein crystal growth. The quality of a crystal is often linked to the number of crystals formed (a few large crystals versus many microcrystals), size (larger is better), and appearance (optically clear, sharply faceted crystals are best). However, any true measure of quality must verify that diffraction correlates with secondary observations.

Secondary measurements of crystal quality (other than the actual X-ray diffraction experiment itself) have been proposed to evaluate protein crystals as well as provide insight into the cause of any imperfection. Recent studies have shown that polarized Raman spectra can be used to detect the relative alignment of certain chemical bonds (i.e. disulfide bridges) within the protein crystal and, thus, crystal perfection (51). In addition, growth and etching studies in an interfero-

metric microscope can reveal defects on the surface of a crystal (70, 93). X-ray topographic studies (26, 83) provide a direct visualization of growth-induced defects and subdomains within the macroscopic crystal. Such defects lead to broadening of the reflection widths. Because the integrated intensity of a given reflection is fixed by the molecular structure, increased width necessarily implies reduced peak height. For high-resolution peaks, the peak height must be significantly above the background signal, and increased width often results in an undetectable reflection.

Ultimately, the direct measurement of diffraction intensity is the best indicator of relative crystal quality. The following diffraction characteristics are typically assessed: (*a*) absolute resolution limit {resolution at which relative intensity above background signal [I/σ(I)] drops below 2.0}; (*b*) diffraction intensity fall-off as a function of resolution; and (*c*) crystal mosaicity. The quantity, quality, and resolution limit of diffracted intensities are the limiting factors for precision of a crystallographic study. Groups comparing the properties of space-grown vs Earth-grown crystals have concentrated on analyzing diffraction intensities as a function of resolution. Relative Wilson plots have been used to make pairwise comparisons of data sets measured from different crystals (23–25). All of these studies have emphasized the importance of collecting three-dimensional data sets for comparison.

The relative intensity (I) of diffracted reflections above the background signal [$\sigma(I)$] is a function of the number of molecules in the diffraction condition. Misalignments or defects in the crystal can effectively reduce the number of molecules in diffracting condition at a particular crystal/source/detector geometry. For low-resolution reflections, such misalignments are indicative of structural misalignments. The intensity is expected to fall off with increased resolution owing to the "misalignment" caused by simple thermal motion within the molecule. An example of diffraction intensity fall-off is shown in Figure 4. Two different growth conditions are compared in this figure. Apparently, crystals grown under microgravity conditions are significantly better than those grown on Earth. The influence of microgravity will be discussed in more detail below. Once the diffracted intensity declines to a value only slightly above the background {typically $[I/\sigma(I)] = 2.0$}, the resolution limit of the crystal is reached. Increasing X-ray intensity and detector sensitivity will not significantly increase the quality of the resulting diffraction data. Any further improvements must come from improved crystal quality.

The physical source of the imperfections within a given protein crystal is usually modeled as a collection of subdomains—each individually perfect—which are slightly misaligned. Thus the mosaic of such domains leads to misalignments and a slight variation in the geometry required to bring each subdomain into the diffracting condition. The mosaicity of a crystal is quantified by the measurement of rocking width (or mosaicity) of the crystal's reflections. A low value (~0.1–0.3°) is usually desirable, but the value can vary considerably from crystal to crystal. Although variation of mosaicity can often be tolerated for a

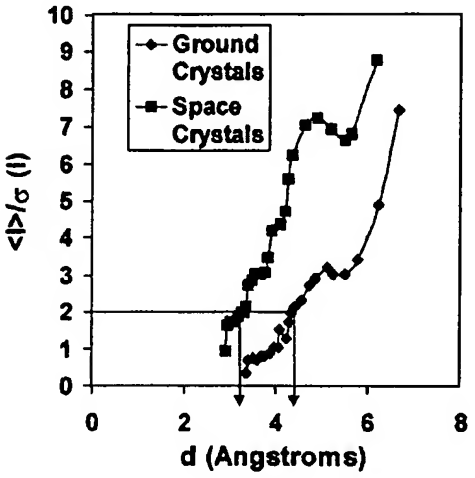

FIGURE 4 Comparison of human serum albumin crystals grown on Earth and in microgravity. (Adapted from Reference 23.) A considerable improvement in resolution is achieved by growing crystals in microgravity, as indicated by the arrows (4.2 Å for Earth-grown versus 3.0 Å for microgravity-grown crystals).

given diffraction experiment, the success of very demanding X-ray experimental procedures, such as Laue time-resolved diffraction, depends critically on having a low crystal mosaic spread (44, 82). Snell et al (82) have advocated this approach to measuring crystal quality for many years, and it is now becoming a standard quality assessment tool. Such measurements demand a highly collimated and monochromatic beam, which is usually not available in home laboratories, but is available at synchrotron sources. An accurate assessment of mosaicity requires the use of synchrotron radiation. Figure 5 shows a comparison of the rocking curves for two crystals grown under the same solution conditions on Earth and in microgravity. Note the dramatic increase in peak intensity observed with the decrease in mosaicity for the microgravity crystal. The measurement of such rocking curve widths along with intensity fall-off provides the most meaningful assessment of crystal quality for the purposes of structural biology studies.

VARIABLES AFFECTING CRYSTAL QUALITY

As mentioned earlier, there are many more similarities between protein crystal growth and small-molecule crystal growth than there are differences (77). This observation has only recently been accepted by the protein crystal growth community. Thus, the sources of crystal imperfection in protein crystals are typically related to composition (impurities) and transport phenomena, especially mass transport to the surface of the growing crystal.

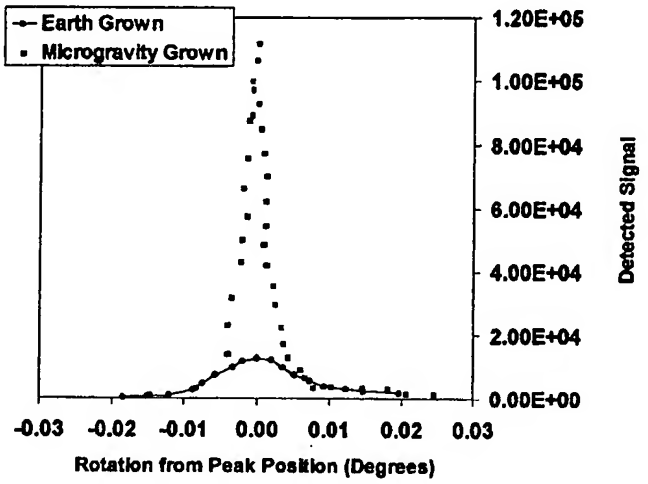

FIGURE 5 Rocking curves measured for lysozyme grown on Earth versus in microgravity. (Adapted from Reference 82.) Note that the Earth-grown crystal displays a much broader peak (~0.0130°) which results in a lower peak intensity than that of the microgravity-grown crystal (mosaicity ~0.0023°) because the integrated areas must, by definition, be equal for a given reflection hkl.

Impurity Incorporation and Mass Transport

Most protein chemists consider a protein that is 95% pure to be highly purified. However, it is well documented that defect formation occurs at much lower concentrations; even sub-ppm levels of an impurity can affect the crystal growth (6). Indeed, the role of impurities in protein crystal defect formation has recently been clearly established. Thomas et al (89) carefully analyzed the most widely studied protein, lysozyme, for sources of impurity. Depending on the source of the protein, the impurities ranged from 1.5% to 5.7% by weight. The purification of lysozyme to a purity of >99.9% was achieved by standard ion exchange liquid chromatography. The resulting crystals were shown to be less sensitive to solution perturbations (temperature in this case), with a resultant reduction in defect density in the crystal. Composition of the protein solution is only part of the picture. The relative rates of mass transfer of impurities and protein play an important role in determination of a protein crystal's quality. In addition, the propensity of an impurity to be included in the protein crystal (partitioning coefficient) also affects the overall impact of such impurities. All of these factors are important, and all can be minimized by proper purification of the initial protein solution.

The Role of Gravity

The role of gravity in protein crystal quality is still not clearly delineated. In general, significant improvements have been documented for some, but not all, crystals grown in the microgravity of space (23–25). The obvious explanations include reduced convective transport, minimal contact of the growing crystal with container surfaces, and elimination of crystal sedimentation. It is generally accepted that crystals tend to grow much more slowly in microgravity. This supports the hypothesis that convective mass transport is playing a role in the rate of crystallization, because a reduction in convection should lead to a concomitant reduction in crystal growth rates. In addition, the accepted measurements of crystal quality (see Figures 4 and 5) indicate that something significant is occurring. Rosenberger et al (76) have discussed the possible roles of convection on crystal quality and have been able to relate defect incorporation to transport regime (i.e. surface kinetic control vs transport control) by using a coupled regime transport model. This model predicts that growth imperfections are maximized when in the mixed regime (neither kinetics nor transport dominate). Thus, either increased convection or decreased convection should improve crystal quality. This model nicely predicts that only those systems in or near the mixed regime on Earth will see an improved quality in the microgravity in space. This prediction is consistent with the observation that some, but not all, crystals show significant quality improvement when grown in microgravity. Of course, the model also predicts that increased convection (mixing) should be an alternative to microgravity, and most crystallographers would shudder at the thought of trying to grow these very fragile crystals under such a high-shear environment.

One of the more obvious roles of microgravity in improving protein crystal quality is the minimization of sedimentation. Malkin et al (66) have noted that a variety of "junk" falls onto the surface of the growing protein crystals and is incorporated into the macroscopic crystal itself. Figure 6 shows a time-lapse study of such an event. In this case, the sedimenting particle is a microcrystal of the same protein. This microcrystal has its own underlying lattice, which will not be perfectly aligned with growing large crystals and lead to a noticeable deterioration of the overall crystal quality.

Active and Passive Control of Crystallization

It has been a mantra of protein crystal growers, "The slower you grow the crystal, the better." This heuristic is supported by both the mixed-regime model of Rosenberger et al (76) and microgravity experiments. If the system is placed in a state in which the transport rate is limiting (solution concentration close to equilibrium concentration), the crystals should grow to a large size with a minimum of defects, provided that impurities are minimized. Devices and techniques to achieve such reduced growth rates are under development in several labs.

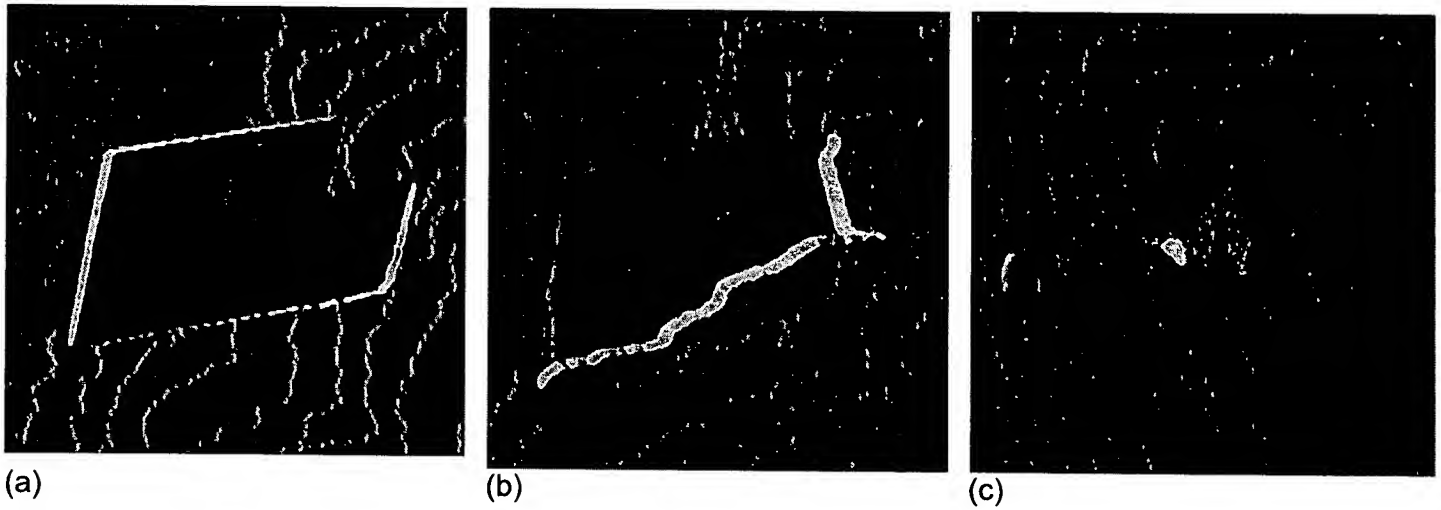

FIGURE 6 Contamination of a protein crystal can occur by sedimentation of impurities or microcrystals onto the growing surface of the crystal. In this set of AFM images (66), a microcrystal is completely incorporated into a growing canavalin crystal. (*a*) Initial settling of microcrystal. (*b*) 900 s after (*a*) occurred, the microcrystal is partially incorporated into the macroscopic crystal. (*c*) 1500 s after (*a*) occurred, the microcrystal is completely engulfed by the growing macroscopic crystal.

Luft & DeTitta (61) have focused on developing devices that require no intervention after the initiation of the experiment. The advantage of simplicity and reproducibility speaks in favor of such an approach; however, the inability to control many aspects of the experiment after it starts may lead to situations in which effective control of the growth rate is not sufficiently responsive. Luft and DeTitta have clearly demonstrated the effectiveness of the passive device in a variety of experiments. The devices have included a variation of the vapor diffusion cell with a simplified sealing mechanism, called HANGMAN (59, 60), a variable diffusion path length cell for vapor diffusion experiments, called a Z/3 cell (1, 58), and a microbatch thermal gradient system (62, 63). The use of these devices is complemented with a substantial effort to model and predict the behavior of the devices.

Recently, a variety of active control devices have been developed by several investigators (8, 80, 95), most relying on temperature manipulations to control the rates of growth. Temperature affects primarily the solubility of the protein and, thus, its supersaturation. For a "normal" system, a reduction in temperature will increase the supersaturation and, in turn, the rate of crystal nucleation and growth. By judicious changes in the temperature as a function of time in a batch experiment, the nucleation event can be separated from subsequent crystal growth. Further, the rate of growth can be controlled to values set by the experimentalist, rather than being set by the initial conditions of the experiment as in the passive experiments described above. Bray et al (8) and Ward et al (95) use active feedback (i.e. a light-scattering signal) to determine the onset of nucleation and then rely on scanning a variety of temperature profiles to arrive at an optimum temperature control algorithm. Bray et al (8) have also developed a device that uses dry nitrogen in a vapor-diffusion cell to control the supersaturation in systems in which temperature is not an effective control action. Schall et al (80) have focused on characterizing key features of the system including critical nucleation temperatures, solubility, and growth rates to arrive at a predetermined temperature control algorithm. Active control can dramatically improve crystal quality by allowing the growth of crystals under conditions of constant growth rate, albeit relatively slow rates. For example, the uncontrolled crystallization of lysozyme from NaSCN solutions often results in clustered crystals, which would be unacceptable for diffraction study, yet controlled growth can yield a single, high-quality crystal (see Figure 7).

SUMMARY

Protein crystallization is an underappreciated field of study. As pointed out in this article, much of structural biology hinges on the ability to crystallize a protein molecule. Applications and implications in fields beyond protein crystallography abound, and fundamental advances in our understanding of protein crystallization will have significant impact in areas such as pharmaceutical process design, pro-

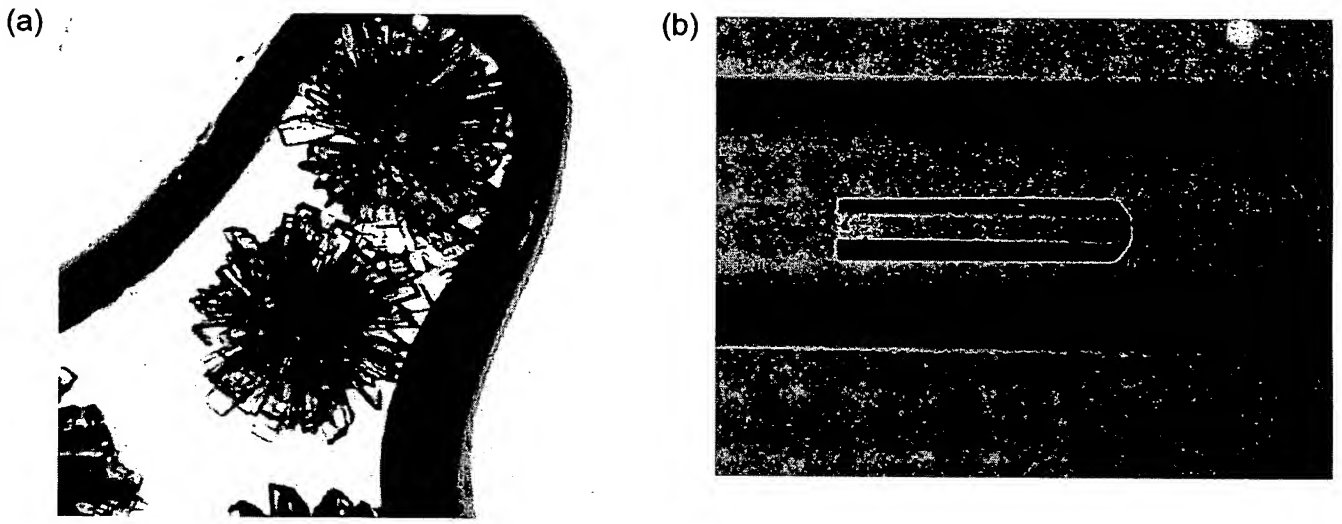

FIGURE 7 Crystals of lysozyme grown from NaSCN solution by (*a*) isothermal and (*b*) temperature-programming techniques. Certainly crystals such as those in (*a*) are very difficult, if not impossible, to use for X-ray diffraction work, whereas crystals as shown in (*b*) typically yield high-quality data.

tein folding, tissue preservation, inclusion body formation, and the origin of ß-amyloid plaques associated with Alzheimer's disease. Advances will continue as the rate-limiting step of structural determinations, protein crystallization, becomes ever more the focus of the structural biology community.

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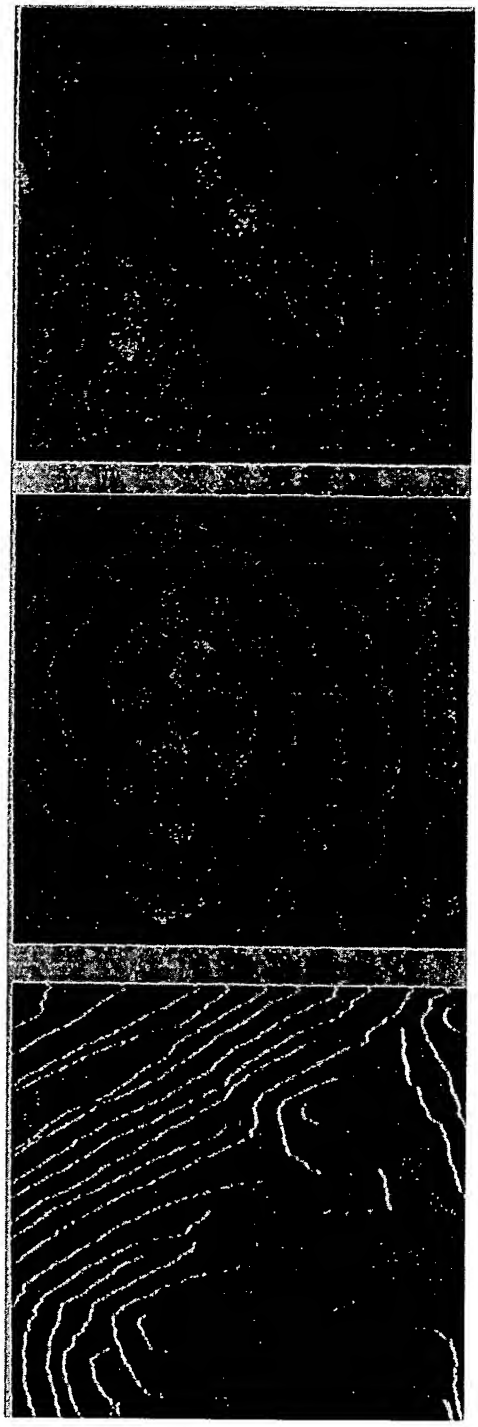

Figure 3a Screw dislocations on the surface of a growing crystal of the protein canavalin as detected by atomic force microscopy (66). Both left- and right-handed screw dislocations can be seen on this crystal face.

Figure 3b Even double-screw dislocations can be detected on the surface by atomic force microscopy.

Figure 3c Spiral growth steps are evident on the surface of a thaumatin crystal.